RESEARCH ARTICLE

# Stored-product insects carry antibiotic-resistant and potentially virulent enterococci

Lakshmikantha H. Channaiah[1], Bhadriraju Subramanyam[1], Leland J. McKinney[1] & Ludek Zurek[2,3]

[1]Department of Grain Science and Industry, Kansas State University, Manhattan, KS, USA; [2]Department of Entomology, Kansas State University, Manhattan, KS, USA; and [3]Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS, USA

**Correspondence:** Bhadriraju Subramanyam, Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506-2201, USA. Tel.: +1 785 532 4092; fax: +1 785 532 7010; e-mail: sbhadrir@k-state.edu

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## Abstract

A total of 154 enterococcal isolates from 95 stored-product insects collected from a feed mill, a grain storage silo, and a retail store were isolated and identified to the species level using PCR. *Enterococcus casseliflavus* represented 51% of the total isolates, followed by *Enterococcus gallinarum* (24%), *Enterococcus faecium* (14%), *Enterococcus faecalis* (7%), and *Enterococcus hirae* (5%). Many isolates were resistant to tetracycline (48%), followed by streptomycin (21%), erythromycin (14%), kanamycin (13%), ciprofloxacin (12%), ampicillin (4%), and chloramphenicol ( < 1%). Enterococci carried genes coding for virulence factors, including the gelatinase gene *gelE* (26% of isolates), an enterococcal surface protein gene *esp* (1%), and the cytolysin gene *cylA* (2%). An aggregation substance (*asa1*) gene was detected in six out of 10 *E. faecalis* isolates and five of these were positive for the aggregation substance. Enterococci were positive for hemolytic (57% of isolates) and gelatinolytic (23%) activity. The filter-mating assay showed that the tetracycline resistance gene, *tetM*, was transferable among *E. faecalis* by conjugation. These data demonstrated that stored-product insects can serve as potential vectors in disseminating antibiotic-resistant and potentially virulent enterococci.

## Introduction

Stored-product insects are cosmopolitan in distribution (Hagstrum & Subramanyam, 2009) and are adapted to infesting raw and processed cereal products, posing a constant threat to stored-food commodities worldwide (Sinha & Watters, 1985). Numerous stored-product insect species are associated with stored-grain elevators, feed mills, and retail stores (Rilett & Weigel, 1956; Roesli *et al*., 2003; Larson *et al*., 2008a). These pests cause significant quantitative and qualitative losses to the multibillion dollar grain, food, feed, and retail industries each year through their feeding and product adulteration. In addition, several stored-product insects have been reported to harbor potentially pathogenic bacteria. For example, the darkling beetle, *Alphitobius diaperinus* (Panzer), a cosmopolitan general stored-product pest from poultry brooder houses was reported to contain *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp. (Husted *et al*., 1969; Harein *et al*., 1970; Bates *et al*., 2004; Templeton *et al*., 2006), *Micrococcus* spp., *Streptococcus* spp., and *Bacillus subtilis* (De Las Casas *et al*., 1972). *Escherichia intermedia*, *Proteus rettgeri*, *Proteus vulgaris*, *B. subtilis*, *Serratia marcescens*, *Streptococcus* spp., *Micrococcus* spp., and members of the *Klebsiella–Aerobacter* group were isolated from laboratory colonies of the granary weevil, *Sitophilus granarius* (L.) (Harein & De Las Casas, 1968).

However, very few studies (Yezerski *et al*., 2005; Larson *et al*., 2008b; Channaiah *et al*., 2010) have addressed the importance of stored-product insects carrying enterococci. To our knowledge, there are no studies addressing the antibiotic resistance and virulence profiles of enterococci associated with stored-product insects. Also, there are no studies that show whether enterococci isolated from stored-product insects can disseminate antibiotic resistance genes to other enterococci.

Enterococci, once considered harmless commensal Gram-positive bacteria, have emerged recently as important reservoirs for antibiotic resistance, accounting for many hospital-borne infections worldwide (Huycke *et al*., 1998; Upadhyaya *et al*., 2009). Enterococci are opportunistic pathogens that have become increasingly important in recent years due to the development and transmission of antibiotic resistance traits (McCormick *et al*., 2008; Ogiera

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& Serror, 2008). In our previous studies, we reported antibiotic-resistant enterococci from stored-product insects collected from feed mill environments (Larson *et al.*, 2008b) and also the vector potential of the red flour beetle for *Enterococcus faecalis* OG1RF:pCF10 (Channaiah *et al.*, 2010). Also, enterococci have been isolated from feed samples in Sweden, Spain, United Kingdom, and United States (Schwalbe *et al.*, 1999; Kidd *et al.*, 2002; Kuhn *et al.*, 2003). We hypothesize that stored-product insects associated with storage environments are able to acquire and serve as potential vectors in disseminating antibiotic-resistant and virulent enterococci. The main objective of this study was to determine the prevalence, number of enterococci per insect, and diversity of antibiotic-resistant and potentially virulent enterococci associated with stored-product insects collected from a feed-manufacturing facility, grain storage silo, and retail store. Additionally, the ability of these enterococcal isolates in transferring antibiotic resistance traits to other enterococci was also examined.

## Materials and methods

### Insect collection

A total of 228 stored-product insects were collected from a feed mill, a grain storage silo holding wheat, and a retail store in Kansas during March through August 2006. Stored-product insect adults in the feed mill were collected using commercial food and pheromone-baited pitfall traps (Trécé Inc., Adair, OK), whereas insects in a grain silo were collected using a perforated plastic probe trap (Subramanyam & Hagstrum, 1995) inserted below the grain surface. Adult insects in infested wheat flour were shipped to us from a retail store. Insects in the flour were separated using an 840-μm opening brass sieve (Seedburo Equipment Company, Batavia, IL). All insects were collected using sterile forceps and individually placed into sterile plastic vials, labeled, and transported to the laboratory for further microbial analysis.

### Isolation, enumeration, and identification of enterococci using PCR

Individual insects of each species were identified to the species level and surface sterilized with 10% sodium hypochlorite and 70% ethanol (Zurek *et al.*, 2000), homogenized in phosphate-buffered saline (pH 7.2; MP Biomedicals, Solon, OH), and drop-plated on mEnterococcus agar (mENT; Difco Laboratories, Detroit, MI). Plates were allowed to dry and then placed in an incubator at 37 °C for up to 48 h. After incubation, the CFUs were recorded to determine the number of enterococci per insect. Up to three presumptive enterococcal colonies with different colony morphologies from each insect sample were isolated on trypticase soy broth agar (TSBA; Difco Laboratories), incubated at 37 °C for 24 h, and stored at 4 °C for further analysis.

The presumptive identities of enterococcal isolates were confirmed to the genus level by the esculin hydrolysis test using Enterococcosel broth (Difco Laboratories). All enterococcal isolates were grown in esculin broth using 96-well plates (Fisher Scientific, Pittsburg, PA), and incubated at 44.5 °C for 6 h. All positive enterococcal isolates were stabbed in TSBA (0.3% agar) in 2.0-mL vials and stored at 4 °C.

Multiplex PCR was used to identify four common species of enterococci, including *E. faecalis* (*$ddl_{E.\ faecalis}$*), *Enterococcus faecium* (*$ddl_{E.\ faecium}$*), *Enterococcus casseliflavus* (*vanC2/C3*), and *Enterococcus gallinarum* (*vanC1*) (Dutka-Malen *et al.*, 1995; Kariyama *et al.*, 2000). *Enterococcus mundtii* ATCC 43186 served as the negative control. A single PCR was run to identify *Enterococcus hirae* (*mur-2*) using the specific primer set (Arias *et al.*, 2006). *Enterococcus hirae* ATCC 8043 was used as the positive control. The enterococcal isolates, screened by multiplex and single PCR using specific primers, resulted in the identification of 90 (58%) isolates. The unidentified isolates (42%) were identified by amplifying the *sodA* (superoxide dismutase) gene using PCR (Poyart *et al.*, 2000) by sequencing (Genomics Center, University of California, Davis, CA) and by BLAST in the National Center for Biotechnology Information GenBank database. All identified isolates were characterized further by screening for antibiotic resistance and virulence determinants by PCR and phenotypic tests.

### Phenotypic screening of enterococci for antibiotic resistance

The protocols used to screen isolates for antibiotic resistance were based on the established guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2000). The antibiotics selected for this screening were based on their usage in animal agriculture. Enterococcal isolates were screened for antibiotic sensitivity using the disk diffusion method on Muller–Hinton Agar (Difco Laboratories) for seven antibiotics: ampicillin (10 μg), ciprofloxacin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), vancomycin (30 μg), and gentamicin (120 μg). Susceptibility of isolates to kanamycin (2000 $\mu g\,mL^{-1}$) and streptomycin (2000 $\mu g\,mL^{-1}$) were assessed by the agar dilution method using brain–heart infusion agar (BHI; Difco Laboratories). *Enterococcus faecalis* ATCC 19433 was used as the quality control strain.

### Genotypic screening of enterococci for antibiotic resistance and virulence determinants

Identified enterococcal isolates were screened for tetracycline and erythromycin resistance genes using multiplex and single PCR (Macovei & Zurek, 2006). Tetracycline resistance genes were divided into three groups. The group I multiplex


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PCR consisted of *tetA*, *tetC*, and *tetQ* genes, while group II consisted of *tetM*, *tetS*, tet*K*, and *tetO* genes (Ng *et al.*, 2001; Macovei & Zurek, 2006). A single PCR was used to detect the *tetW* gene (Aminov *et al.*, 2001). The *ermB* gene was used to screen all isolates for erythromycin resistance using single PCR (Sutcliffe *et al.*, 1996). Similarly, enterococcal isolates were screened for four important virulence determinants, namely *asa1* (aggregation substances), *cylA* (cytolysin), *esp* (enterococcus surface protein), and *gelE* (gelatinase) genes using multiplex PCR (Vankerckhoven *et al.*, 2004).

### Phenotypic screening of enterococci for gelatinase, hemolysin, and aggregation substance

The Todd–Hewitt broth (THB; Difco Laboratories) with 2% milk powder was used for the detection of gelatinase activity. All enterococcal isolates were streaked on THB plates and incubated at 37 °C for 24 h. After incubation, the plates were examined for a zone of clearance surrounding the colonies (Gevers *et al.*, 2003). Columbia blood agar (Difco Laboratories) with 5% human blood was used to assess cytolysin (β-hemolysis) activity. All the enterococcal isolates were streaked and incubated at 37 °C for 48–72 h. Clear zones around the colonies (β-hemolysis) were considered positive for cytolysin activity. The *E. faecalis* strain MMH-594 was used as a positive control for both gelatinase and cytolysin activity tests.

The clumping assay for enterococcal aggregation substances was carried out exclusively using *E. faecalis* isolates (Dunny *et al.*, 1978). *Enterococcus faecalis* JH2-2 was used for cCF10 peptide formation. *Enterococcus faecalis* JH2-2 was grown in THB at 37 °C for 18 h. The supernatant containing pheromone (peptide) was collected by centrifuging at 9.3 ***g*** for 10 min followed by autoclaving for 15 min. The test isolates were grown in THB (5 mL) at 37 °C for 18 h, after which 1 mL of *E. faecalis* JH2-2 supernatant was added to each culture and incubated at the same temperature overnight in an incubator with a shaker (Excel E24 Incubator Shaker Series, Edison, NJ). Isolates after incubation were considered positive if clumping or aggregation of cells was observed visually or under a microscope (Dunny *et al.*, 1978). *Enterococcus faecalis* OG1RF:pCF10 was used as the positive control.

### Screening enterococci for mobile genetic elements (Tn*916*/*1545* family transposons)

All enterococcal isolates identified were screened for the integrase gene (*int*) for the detection of Tn*916*/*1545* family conjugative transposons (Gevers *et al.*, 2003) that frequently carry the tetracycline resistance gene *tetM*. *Enterococcus faecalis* OG1RF:pCF10 was used as a positive control.

### Conjugation assay

Broth mating and filter matings were carried out as described by Ike *et al.* (1998) and Tendolkar *et al.* (2006) to study the mobility of the *tetM* gene. Both assays were performed at a donor and recipient ratio of 1 : 10. *Enterococcus faecalis* (10 isolates), resistant to tetracycline but susceptible to streptomycin, were used as the donor, whereas *E. faecalis* OG1SSp, resistant to streptomycin but susceptible to tetracycline, was used as the recipient.

In broth mating experiments, overnight cultures of donor (0.5 mL) and recipient (5 mL) grown in the BHI broth (Difco Laboratories) were mixed in 5 mL fresh BHI broth and incubated at 37 °C for 4 h, with gentle agitation in an incubator with a shaker. After incubation, 100 μL of the mixed culture was plated on BHI agar plates supplemented with tetracycline (40 mg $L^{-1}$), streptomycin (2 g $L^{-1}$), and plates containing both tetracycline (40 mg $L^{-1}$) and streptomycin (2 g $L^{-1}$). Plates were incubated overnight at 37 °C.

In filter mating experiments, overnight cultures of the donor (0.5 mL) and recipient (5 mL) were mixed, and cells were collected on a 0.2-μm cellulose nitrate membrane filter (Whatman International Ltd, Dassel, Germany) using a filter holder (Fisher Scientific). The filter was placed on BHI agar plates with the bacteria side up and incubated at 37 °C for 16 h. After incubation, the cells from the filter were suspended in 1 mL BHI broth, and appropriate dilutions were plated on BHI agar plates containing tetracycline (40 mg $L^{-1}$), streptomycin (2 g $L^{-1}$), and plates containing both tetracycline (40 mg $L^{-1}$) and streptomycin (2 g $L^{-1}$). Plates were incubated overnight at 37 °C. After incubation, CFUs for broth and filter matings were recorded to determine the transfer frequency, which was expressed as the number of transconjugants per recipient. The presence of the *tetM* gene in the transconjugants was confirmed by PCR as described above.

## Results

### Isolation, enumeration, and identification of enterococci

A total of 95 out of the 228 insects collected were positive for enterococci. The 95 insects represented 11 stored-product insect species. From these 95 insects, 154 enterococcal isolates were isolated (Table 1). Samples from the feed mill contributed to a majority of the species, followed by retail store and grain storage silo. The percentage of stored-product insects positive for enterococci ranged from zero for the small-eyed flour beetle, *Palorus ratzeburgii* (Wissmann), and the maize weevil, *Sitophilus zeamais* Motschulsky, to 50% for the darkling beetle, *A. diaperinus* (Panzer), the lesser grain borer, *Rhyzopertha dominica* (F.), and the foreign grain beetle, *Ahasverus advena* (Waltl.), followed by

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**Table 1.** Prevalence and concentration of enterococci from adult stored-product insect species collected from three storage environments

| Insect species | No. of insects | No. of insects positive for enterococci (%) | No. of enterococcal isolates (%) | No. of enterococci per insect |
|---|---|---|---|---|
| Feed mill | | | | |
| Red flour beetle | 75 | 34 (45.3) | 44 (28.5) | 41 ± 1.0 |
| Confused flour beetle | 70 | 30 (42.8) | 39 (25.3) | 38 ± 1.0 |
| Warehouse beetle | 18 | 8 (44.4) | 16 (10.4) | 40 ± 2.0 |
| Rusty grain beetle | 10 | 3 (30.0) | 8 (5.2) | 37 ± 3.0 |
| Lesser grain borer | 8 | 4 (50.0) | 8 (5.2) | 36 ± 2.0 |
| Drugstore beetle | 7 | 3 (42.8) | 9 (5.8) | 35 ± 2.0 |
| Darkling beetle | 2 | 1 (50.0) | 2 (1.3) | 35 ± 5.0 |
| Foreign grain beetle | 2 | 1 (50.0) | 2 (1.3) | 35 ± 5.0 |
| Small-eyed flour beetle | 1 | 0 (0) | 0 (0) | 0 (0) |
| Maize weevil | 1 | 0 (0) | 0 (0) | 0 (0) |
| Total | 194 | 84 (43.3) | 129 (83.7) | 37 ± 8.0 |
| Grain storage silo | | | | |
| Red flour beetle | 6 | 2 (33.3) | 3 (1.9) | 23 ± 3.0 |
| Confused flour beetle | 4 | 1 (25.0) | 2 (1.3) | 20 ± 0.0 |
| Total | 10 | 3 (30.0) | 5 (3.3) | 23 ± 1.0 |
| Retail store | | | | |
| Sawtoothed grain beetle | 24 | 8 (33.3) | 20 (13.0) | 21 ± 1.0 |
| Grand Total | 228 | 95 (41.6) | 154 (100.0) | 27 ± 5.0 |

the red flour beetle, *Tribolium castaneum* (Herbst), and the warehouse beetle, *Trogoderma variabile* Ballion. Of all the insect species, the red flour beetle, *T. castaneum*, yielded 31% of the total enterococcal isolates followed by the confused flour beetle, *Tribolium confusum* (Jacquelin du Val) (23%). Enterococcal loads in insects that tested positive ranged from 20 to 41 CFU per insect, with greater loads found in insects from the feed mill compared with those from retail stores and grain storage silo.

The majority of the isolates belonged to *E. casseliflavus*, followed by *E. gallinarum*, *E. faecium*, *E. faecalis*, and *E. hirae* (Table 2). All *E. faecium* and *E. faecalis* isolates were from stored-product insects associated with the feed mill.

## Antibiotic resistance profiles of enterococci

Enterococcal isolates were resistant to tetracycline, followed by resistance to streptomycin, erythromycin, kanamycin, ciprofloxacin, ampicillin, and chloramphenicol (Table 2). All enterococcal isolates were susceptible to vancomycin and gentamicin. Twenty-eight out of 154 enterococcal isolates were resistant to more than one antibiotic. Specifically, five out of 10 *E. faecalis* isolates, nine out of 22 *E. faecium*, six out of 37 of *E. gallinarum*, seven out of 78 of *E. casseliflavus*, and one out of seven *E. hirae* were resistant to more than one antibiotic. Resistance to commonly used tetracycline and streptomycin antibiotics was quite common (12 out of 28 isolates), followed by resistance to tetracycline and erythromycin (nine out of 28 isolates) and tetracycline and ciprofloxacin (seven out of 28 isolates).

## Prevalence of *tet*, *erm*, and Tn*916*/*1545* family of conjugative transposons

The most common tetracycline resistance genes, *tetM* and *tetO*, were detected from 71 and 25 of the total 154 isolates, respectively (Table 2). The other tetracycline resistance genes *tetA*, *tetC*, *tetQ*, *tetK*, *tetS*, and *tetW* were not detected in any of the isolates. The *tetM* gene was detected most frequently in *E. faecalis*, followed by *E. faecium*, *E. gallinarum*, and *E. casseliflavus*. The *ermB* gene encoding macrolide resistance was detected in 6% of the total isolates. The Tn*916*/*1545* family conjugative transposons was detected in 15 (10%) of the total isolates, and 13 of these isolates carried either *tetM* or *ermB* genes. All isolates that carried Tn*916*/*1545* family conjugative transposons were from *E. faecalis* and *E. faecium* isolated from the red flour beetle, confused flour beetle, and rusty grain beetle collected from the feed mill.

## Screening enterococci for virulence determinants

The genotypic analysis of virulence determinants revealed that out of the 154 enterococcal isolates, 26% had the *gelE* gene, 2% had the *cylA* gene, and 1% had the *esp* gene (Table 3). The *asa1* gene was detected in six out of 10 isolates of *E. faecalis* and five out of 10 *E. faecalis* isolates were positive for the aggregation substance. A total of 23% isolates were positive for gelatinase activity. Among enterococcal species, a majority of *E. faecalis* isolates exhibited the gelatinase activity, while only a few *E. casseliflavus* isolates exhibited the gelatinase phenotype. None of the *E. hirae*


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**Table 2.** Antibiotic resistance profiles and the Tn*916/1545* family of transposons in enterococci isolated from stored-product insects

| | | No. of isolates resistant to an antibiotic* | | | | | | | Tetracycline resistance genes | | Erythromycin resistance gene | Transposon family Tn*916/1545* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Enterococci | No. of isolates (%) | Amp | Cip | Chl | Ery | Kan | Str | Tet | *tetM* | *tetO* | *ermB* | |
| *E. faecalis* | 10 (6.5) | 2 | 4 | 0 | 5 | 5 | 0 | 10 | 10 | 7 | 5 | 10 |
| *E. faecium* | 22 (14.3) | 2 | 8 | 1 | 11 | 10 | 13 | 16 | 18 | 10 | 3 | 5 |
| *E. gallinarum* | 37 (24.0) | 0 | 2 | 0 | 3 | 3 | 9 | 19 | 17 | 3 | 1 | 0 |
| *E. casseliflavus* | 78 (50.6) | 2 | 3 | 0 | 3 | 2 | 9 | 26 | 24 | 5 | 1 | 0 |
| *E. hirae* | 7 (4.5) | 0 | 1 | 0 | 0 | 0 | 2 | 3 | 2 | 0 | 0 | 0 |
| Total | 154 | 6 | 18 | 1 | 22 | 20 | 33 | 74 | 71 | 25 | 10 | 15 |
| Percentage of total | | 3.8 | 11.6 | 0.7 | 14.1 | 12.8 | 21.2 | 48.0 | 46.1 | 16.0 | 6.4 | 9.5 |

*All five enterococcal species were susceptible to vancomycin and gentamicin.
Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Erm, erythromycin; Kan, kanamycin; Str, streptomycin; Tet, tetracycline.

**Table 3.** Number of enterococcal isolates from stored-product insects showing phenotypic and genotypic virulence profiles

| | | Virulence genes | | | | Phenotypic virulence profiles | | |
|---|---|---|---|---|---|---|---|---|
| Enterococci | No. of isolates (%) | *gelE* | *asa1** | *esp* | *cylA* | Gelatinase | β-Hemolysis | Aggregation substance* |
| *E. faecalis* | 10 (6.5) | 8 | 6 | 2 | 2 | 8 | 6 | 5 |
| *E. faecium* | 22 (14.3) | 12 | – | 0 | 1 | 5 | 5 | – |
| *E. gallinarum* | 37 (24.0) | 10 | – | 0 | 0 | 17 | 27 | – |
| *E. casseliflavus* | 78 (50.6) | 10 | – | 0 | 0 | 5 | 48 | – |
| *E. hirae* | 7 (4.5) | 0 | – | 0 | 0 | 0 | 2 | – |
| Total | 154 | 40 | 6 | 2 | 3 | 35 | 88 | 5 |
| Percentage of total | | 26.0 | 60.0 | 1.2 | 1.9 | 22.7 | 57.1 | 50.0 |

**E. faecalis* only.

isolates was positive for the gelatinase phenotype. A total of 57% of the total enterococcal isolates were positive for β-hemolytic activity (Table 3). Hemolytic activity was prominent in *E. casseliflavus*, followed by *E. gallinarum*, *E. faecalis*, *E. faecium*, and *E. hirae*.

### Conjugation assays

The broth mating assay did not yield any transconjugants. The filter mating assay clearly showed that *E. faecalis* isolated from stored-product insects could transfer tetracycline resistance encoded by *tetM* to the recipient strain *E. faecalis* OG1SSp. Out of the 10 *E. faecalis* isolates tested, three isolates showed the frequency between $5.7 \times 10^{-5}$ and $8.1 \times 10^{-7}$ transconjugants per recipient. The remaining six isolates showed a frequency $> 10^{-8}$ transconjugants per recipient, which could also be due to spontaneous mutation.

## Discussion

The number of individuals positive for enterococci was greater for insects collected from feed mill, followed by retail store and grain store, and a majority of insects that tested positive were red and confused flour beetles. This trend is related to more individuals of these species examined from the feed mill compared with other habitats. Red and confused flour beetles made up 68% of the total insects isolated, because specific lures for these species were used in the pitfall traps deployed in the feed mill. These two insect species are economically important, especially in grain, flour, and feed mills (Hagstrum & Subramanyam, 2006; 2009).

The presence of *E. casseliflavus, E. gallinarum*, and *E. hirae* in stored-product insects is expected, as these stored-product insects feed mainly on grain and grain-based products, including feed (Franz *et al*., 2003). Although *E. gallinarum*, *E. casseliflavus*, and *E. hirae* are infrequently isolated from clinical settings, they can cause a wide variety of invasive infections in humans, especially in immunocompromised or chronically ill patients (Gordon *et al*., 1992; Reid *et al*., 2001; Prakash *et al*., 2005). The presence of antibiotic resistance genes in *E. casseliflavus*, *E. gallinarum*, and *E. hirae* cannot be ignored as enterococci are capable of transferring their resistance genes, within the same species, to other enterococci (Moellering, 1992) and to bacteria of clinical significance (Leclerq *et al*., 1989; Noble *et al*., 1992). The source of contamination of stored-product insects with *E. faecalis* and *E. faecium* is unknown and warrants further study. However, the low prevalence of antibiotic resistance

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and virulence genes suggests that the enterococcal contamination may be environmental and not of clinical origin.

Isolates of *E. faecalis* and *E. faecium* were from stored-product insects associated with the feed mill, and the insect species included the red and confused flour beetles, rusty grain beetle, and warehouse beetle. These species are commonly associated with feed mills in the Midwestern United States (Larson *et al*., 2008a; Hagstrum & Subramanyam, 2009). Animal byproducts such as meat meal, blood meal, bone meal, feather meal, egg-shell meal, and fish meal are common ingredients used in animal feed, and may be the source(s) of *E. faecalis* and *E. faecium* in feed samples (Cox *et al*., 1983; Okoli *et al*., 2006; Sapkota *et al*., 2007). In previous studies from our laboratory (Larson *et al*., 2008b; Channaiah, 2009), we isolated antibiotic-resistant and virulent enterococci from animal feed samples collected from six feed mills located in the Midwestern United States. It is plausible that stored-product insects feeding on contaminated feed may have acquired *E. faecalis* and *E. faecium* in their digestive tract. It is still unclear as to how the feed products or insects are exposed to enterococci, and this requires a systematic evaluation of various sources of enterococci in environments being sampled and changes in enterococcal loads in insects on temporal and spatial scales.

Many enterococcal isolates were resistant to tetracycline, an antibiotic that is commonly used for growth promotion and therapeutic purposes in animal feed (Sapkota *et al*., 2007). Resistance to streptomycin, kanamycin, erythromycin, and ciprofloxacin were also found in enterococci isolated in this study. The aminoglycosides, streptomycin and kanamycin, are commonly used in animal feed to treat intestinal infections. The resistance to erythromycin in enterococci isolated in this study is likely due to the widespread use of erythromycin in animal agriculture for treatment of infections caused by Gram-positive bacteria (Skeeles, 1991; Corpet, 1996). The resistance to ciprofloxacin may be due to the use of a closely related drug, enrofloxacin, in animal feed (Delsol *et al*., 2004). We found a very low percentage of isolates resistant to ampicillin and chloramphenicol, which is expected, as these antibiotics were banned in the United States for use in animal agriculture (Hofacre *et al*., 2001; Dawson, 2005).

Genotypic analysis revealed that most of the tetracycline resistance was likely due to *tetM* followed by *tetO* genes. Similar results were reported by Macovei & Zurek (2006), in which enterococci isolated from houseflies collected from five restaurants exhibited tetracycline resistance through the *tetM* gene. The presence of the *ermB* gene in erythromycin-resistant enterocooci isolated in this study is significant as it confers cross-resistance to other macrolides, lincosamide, and streptogramin type B antimicrobials (Jackson *et al*., 2004).

Our study showed that virulence determinants (*gelE*, *asa1*, *cylA*) are relatively common in enterococcal isolates and can be comparable to the virulence determinants in some clinical isolates (Gilmore *et al*., 2002; Creti *et al*., 2004). The number of isolates with β-hemolytic activity was higher than the presence of *cylA*, indicating the involvement of other cytolysin determinants. The higher prevalence of the *gelE* gene among enterococcal isolates relative to gelatinase activity may be due to the presence of silent genes that are expressed only under *in vivo* conditions (Creti *et al*., 2004). Isolates of *E. faecalis* that contained the *cylA* gene also contained the *asa1* gene. Generally, *cylA* and *asa1* genes are carried on plasmids (such as pAD1) and can be transferred to other enterococcal species (Gilmore *et al*., 2002; Creti *et al*., 2004).

Less than 10% of the enterococcal isolates were positive for the Tn*916*/*1545* family of conjugative transposons that commonly carry *ermB* and *tetM* genes. Further, the PCR confirmation for tetracycline resistance encoded by the *tetM* gene in transconjugants suggested that the *tetM* locus was present on Tn*916*/*1545*. Despite low concentrations of enterococci in stored-product insects in this study, we suggest that stored-product insects, particularly red and confused flour beetles, feeding on contaminated feed or grain-based products are capable of acquiring antibiotic-resistant and virulent enterococci in their digestive tract and may contribute to the rapid dissemination of antibiotic resistance and virulence genes in the environment. Recently, we have shown that the red flour beetle is a competent vector of the *E. faecalis* OG1RF:pCF10 strain and has the capability of transferring antibiotic-resistant and virulent enterococci from contaminated animal feed to sterile feed (Channaiah *et al*., 2010).

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